

eIF4E isoform 2 in *Schizosaccharomyces pombe* is a novel stress-response factor

Marina Ptushkina, Naglis Malys & John E.G. McCarthy⁺

Posttranscriptional Control Group, Department of Biomolecular Sciences, UMIST, Manchester, UK

Cap-binding proteins of the eIF4E family are generally involved in mediating ribosome recruitment to capped mRNA via an interaction with the initiation factor eIF4G. However, *Schizosaccharomyces pombe* has two eIF4E isoforms, one of which (eIF4E2, encoded by *tif452*) has a relatively low affinity for eIF4G. We show that *tif452* is required for specific stress responses. An *S. pombe*, *tif452*Δ mutant manifests slow growth under conditions of nutrient, temperature and salt stress. eIF4E2 shows a distinct subcellular distribution to eIF4E1, the cap-binding factor that is required for mainstream translation. In response to salt stress, the cellular level of eIF4E2 increases, whereas the amount of intact eIF4G decreases, leaving eIF4E2 as the predominant eIF4E isoform in a cell deficient in eIF4G. The presence of eIF4E2 modifies the competence of *S. pombe* ribosomes to translate mRNAs with structured leaders *in vivo*. The *tif452* promoter has putative stress-response (T-rich) motifs, whereas eIF4E2 seems to be a new type of stress-response factor.

Keywords: translation; fission yeast; eukaryotic initiation factors; eIF4E isoform; stress

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INTRODUCTION

Recruitment of eukaryotic ribosomes to the m⁷GpppN cap structure at the 5' end of mRNA is a critical step in the initiation of translation. This function is fulfilled by the cap-binding initiation complex eIF4F, which has two core components, the eukaryotic initiation factors eIF4E and eIF4G (Raught *et al*, 2000). The largest eIF4F component, eIF4G, has binding sites not only for eIF4E but also for eIF3, eIF4A, eIF1, eIF5, the poly(A) binding protein PABP and, at least in vertebrates, the MAPK-activated protein kinase Mnk1 (Lamphear *et al*, 1995; Mader *et al*, 1995; Tarun & Sachs, 1996; Morley *et al*, 1997; Imataka *et al*, 1998; Ptushkina *et al*, 1998; Pyronnet *et al*, 1999; Asano *et al*, 2000; He *et al*, 2003). The eIF4F complex is tethered to the mRNA cap by

the 25 kDa cap-binding protein eIF4E. The large multisubunit complex eIF3, assisted by eIF1 or eIF5 (He *et al*, 2003), forms a bridge between the 40S ribosomal subunit and eIF4F, whereas the binding of eIF4G to PABP may have a role in promoting interaction between the 3' and 5' ends of mRNA. The significance of the interaction between eIF4G and eIF4A is not fully established, but the latter factor (together with eIF4B) catalyses ATP-dependent RNA helicase activity that may promote ribosomal scanning along structured mRNA (Linder, 2003).

We recently demonstrated that there are two eIF4E-type proteins in the fission yeast *Schizosaccharomyces pombe* (Ptushkina *et al*, 1996, 2001). The *S. pombe* eIF4E2 sequence (encoded by the *tif452* gene) is closely related to that of this organism's eIF4E1, showing 52% identity and 59% similarity. There is an N-terminal extension in eIF4E2, and this isoform comprises a total of 243 amino acids with a molecular weight of 28 kDa. An unexpected feature of eIF4E2 is that it has a relatively low affinity for eIF4G (Ptushkina *et al*, 2001), a property that suggests that it might not be involved in the recruitment of ribosomes to the bulk of cellular mRNA. In this paper, we explore the physiological role of eIF4E2, and discover novel functions and properties that distinguish it from the standard type of cytoplasmic cap-binding protein.

RESULTS

*tif452*Δ mutants manifest stress phenotypes

Previous work revealed that *tif452* is a nonessential gene that is not required for normal logarithmic growth in a glucose-containing medium (Ptushkina *et al*, 2001). However, many genes have important roles in the responses of organisms to stress conditions (Siderius & Mager, 1997). We therefore investigated the effects of nutrient, salt and temperature stress on the growth of a haploid *tif452::kanMX6* disruption strain (Fig 1). Slow-growth phenotypes were observed under all three stress conditions, thus demonstrating that eIF4E2 is required for resistance to these stresses in *S. pombe*.

tif452 is moderately upregulated under stress conditions

The observation that eIF4E2 has a role in multiple stress responses raised the question whether *tif452* expression is subject to regulation. Previous work demonstrated changes in eIF4E2 levels at increased growth temperatures (Ptushkina *et al*, 2001). Here, we

¹Posttranscriptional Control Group, Department of Biomolecular Sciences, UMIST, Manchester M60 1QD, UK

⁺Corresponding author. Tel: +44 161 200 8916; Fax: +44 161 200 8918; E-mail: j.mccarthy@umist.ac.uk

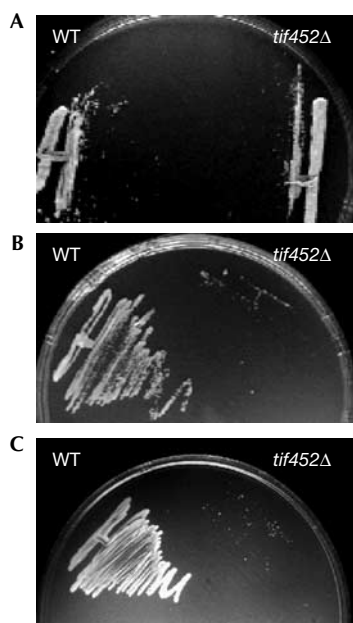


Fig 1 | Disruption of *tif452* results in slow-growth phenotypes under stress conditions. Haploid *S. pombe* CB12 and *tif452::kanMX6* strains were streaked out on YES agar and incubated at 15 °C (A), on YES agar containing 3% glycerol instead of glucose and incubated at 30 °C (B), or on glucose-YES agar containing 0.5 M NaCl (incubated at 30 °C) (C).

observed that the cellular content of eIF4E2 also increases under salt stress conditions (Fig 2A). In contrast, the level of eIF4E1 was unaffected (Fig 2B), whereas the amount of full-length eIF4G was found to be reduced. This was possibly due to proteolytic degradation within the cell, because a number of putative cleavage products (in the M_r range 20–50 kDa) appeared on the gel (Fig 2C). We also investigated whether similar changes in eIF4E2 levels were manifested under nutrient stress conditions (Fig 3). As the cells made the transition between logarithmic and stationary phases, *tif452* mRNA (Fig 3A,C,D) and eIF4E2 protein (Fig 3B) both increased in abundance.

To obtain further information about the mechanism of *tif452* regulation, we characterized the 5' and 3' ends of the *tif452* mRNA and examined the promoter region upstream of this gene. Whereas the 3' untranslated region (UTR) was found to be of a length and constitution that are typical for *S. pombe* mRNAs, the 5'UTR was observed to be relatively long (113 nucleotides; Fig 4). We also observed the presence of novel T-rich promoter motifs (Fig 4) that were previously found to be associated with a subset of stress-regulated genes (Chen *et al*, 2003).

Too much eIF4E2 is growth inhibitory

A cap-binding protein with a specific role in the cellular stress response might be expected to have an optimal range of concentration in which it does not interfere significantly with other mRNA-related functions. We therefore examined in more detail how changes in the level of eIF4E2 influence cellular function. To compare the effects of overexpression of *tif451* and of *tif452*, both genes were tagged with a FLAG-encoding region and inserted into the high-copy-number pREP1 expression vector

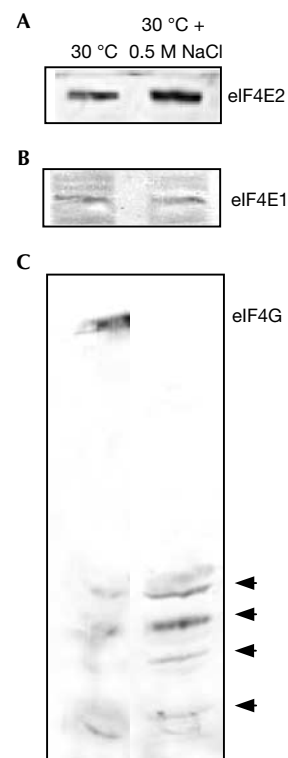


Fig 2 | Upregulation of *tif452* in response to salt stress. *S. pombe* wild-type strain CB12 was grown in rich YES medium at 30 °C with or without 0.5 M NaCl. Cell extracts were prepared from log phase cultures and samples were separated on 12% SDS gels and subjected to western blotting. Membranes were incubated with antibodies versus *S. pombe* eIF4E2 (A), versus *S. pombe* eIF4E1 (B) and versus *S. pombe* eIF4G (C). Arrows in (C) indicate the presence of putative eIF4G cleavage products in the range 20–50 kDa.

(Maundrell, 1993). The FLAG tags allowed direct comparison of the relative amounts of both gene products generated from the expression vector constructs (Fig 5A). Overexpression of *tif452* (using the high-copy-number vector pREP1) in a wild-type strain resulted in severely inhibited growth, whereas overexpression of *tif451* had a barely discernible effect on growth (Fig 5A). Additional experiments in which nontagged *tif451* and *tif452* genes were overexpressed *in vivo* showed the same pattern of inhibition by eIF4E2 but not by excess eIF4E1 (data not shown). Therefore, increasing the concentration of eIF4E2 beyond its normal range in the cell leads to inhibition of cell growth. This may be due to increased competition with eIF4E1 for the mRNA cap, thus compromising the latter factor's ability to mediate ribosome recruitment under normal growth conditions.

eIF4E2 modulates mRNA translation

Earlier investigations using a cell-free translation extract from *S. pombe* indicated that eIF4E2 might serve to enhance the translation of mRNAs bearing structured 5'UTRs (Ptushkina *et al*, 2001). As such behaviour might contribute to the role of eIF4E2 as a stress-response factor, we performed equivalent experiments *in vivo* using a *LUC* reporter gene plasmid. We observed that the absence of eIF4E2 differentially suppresses the translation of a *LUC*

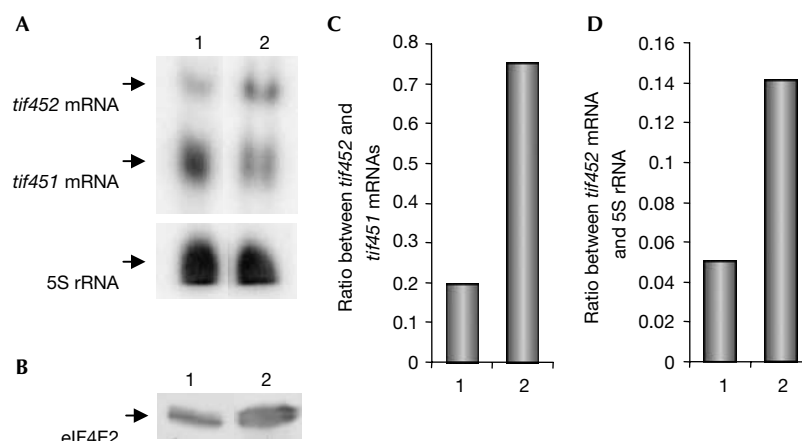


Fig 3 | Upregulation of *tif452* in response to diauxy. As the cells enter the diauxic transition, the abundance of *tif452* mRNA increases relative to the abundance of *tif451* mRNA. A northern blot (A) shows samples from cell cultures at OD₆₀₀ = 0.4 (1) and at OD₆₀₀ = 1.5 (2). The data are quantitated as a ratio of *tif452* signal intensity to *tif451* signal intensity (C) and as the ratio of *tif452* to 5S rRNA (D). A western blot reveals the relative levels of eIF4E2 in the same cultures (B).

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ATTATACTCG TGCATTTCCT ATCAAACAAA TAATTACTG TAAAGCTTTA
AGGCTGATTA TTGACTTTTT ATATATACTT AGCCAATATT TAAGGTATGA
ATGTACAATT ATATTTTACA GGAACAGTGT GTTGAAAGTT GCTTTTGCTT
GGATCTC 5' gta acccaattac gaccaccgca tcttttcagc atccag
ccta gtttacaac tgcctattat aaaaacgttt taagctcttt taata
ttttt gtttttttaa aaaaagcaa atg gca gat gca gaa gac ...
                                M A D A E D
acc cgt atg agt ctt taa tgaTATCAAGTTTCAAGTTTATTTTTT
T R M S L * *
ACTTTTCTTAAGGCTTGGAATATTATAGGGGTGTTCTATTTACGAATGACAATAT
GTTATTGCAATCATTGCCCTCTGTTTGTAGACCTCTAAATACCAAGCTTATT
GAGATGAGATAGATGCCTCTTTACAGTTAGTGATTCGTATGAATAGTTAATT
TGTTATATTGTTGAGCATTTTAATAGGCTAATGGATATAATCTATATTGACC
CTTCTCTTACGTTATAAATATTAATAA... poly(A) 3'

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Fig 4 | UTRs and promoter motifs of *tif452*. The open reading frame (ORF) and flanking regions of the genomic DNA and cDNA were sequenced, and 5' and 3' RACE reactions were performed using the encoded mRNA to map the UTR sequences, the introns and the promoter motifs. The 5'UTR, and the N-terminal and C-terminal parts of the main ORF are shown in lowercase, whereas part of the promoter sequence and the 3'UTR are shown in uppercase.

mRNA, which has an inhibitory stem-loop structure (B3, of stability -17.2 kcal/mol, as described by Oliveira *et al*, 1993) inserted into its 5'UTR (Fig 6). Expression of *tif452* from a pREP42 plasmid in the *tif452Δ* strain restored the relative translation efficiency of B3 mRNA to at least the wild-type level. Thus, a strain of *S. pombe* that lacks eIF4E2 will be less able to generate proteins from mRNAs that have complex leaders. This suggests that modulation of the translational efficiency of a subset of mRNAs may underlie the influence of eIF4E2 on the *S. pombe* stress response.

Localization of eIF4E1 and eIF4E2 in *S. pombe*

As the two eIF4E isoforms have distinct functions in *S. pombe*, we asked the question whether this might be reflected in their subcellular distributions. In initial experiments, we found that antisera generated in rabbits against recombinant eIF4E1 and

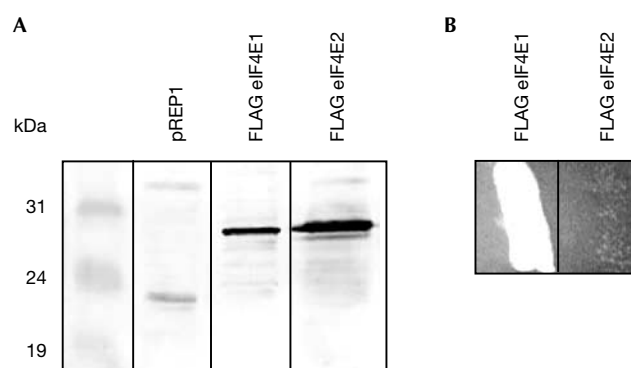


Fig 5 | Phenotype observed with overexpression of *tif452*. The effects of overexpression of *tif451* and *tif452*, respectively, are compared. FLAG-tagged versions of *tif451* and *tif452* were expressed from the pREP1 expression vector in *S. pombe* CB12. Western blotting using anti-FLAG monoclonal antibodies revealed the presence of high levels of the proteins in the respective transformants (A). The control transformant contained only the expression vector pREP1. Overexpression of *tif452* resulted in a slow-growth phenotype, whereas overexpression of *tif451* had no such effect (B).

eIF4E2 proteins were insufficiently specific for use in fluorescence microscopy. We therefore tagged the respective proteins using epitopes that have been shown previously to give high signal-to-noise fluorescence images in *S. pombe* (Salehi *et al*, 2002). Confocal microscopy revealed very different distributions for the two proteins (Fig 7A). Both eIF4E1 and eIF4E2 are almost exclusively cytoplasmic (Fig 7B). Moreover, both proteins show an uneven distribution, with much of the material being located in foci that vary greatly in size. However, as is made particularly evident by the overlay experiment (Fig 7B), there is little colocalization of the two proteins.

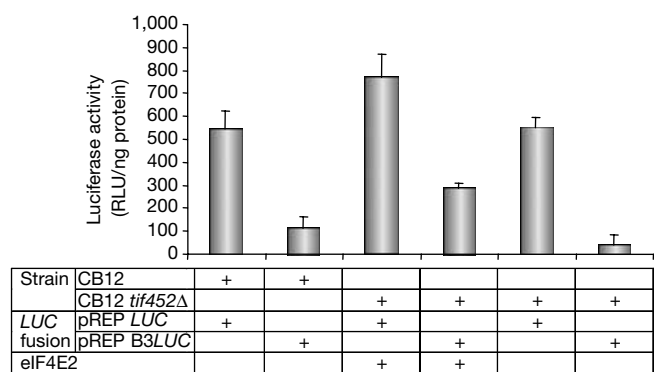


Fig 6 | Modulation of the effect of secondary structure on translation by eIF4E2. LUC reporter plasmids were introduced into *S. pombe* strains. Comparison of the encoded luciferase activities reveals the degree of inhibition of translation by the B3 stem-loop structure, and how this is affected by the disruption or overexpression of *tif452*. The table under the graph indicates which strain and which plasmid(s) were used in each experiment. Error bars on the average values given indicate the standard deviations obtained with eight sets of measurements.

DISCUSSION

S. pombe is not the only organism that possesses multiple species of cap-binding protein (Ptushkina et al, 2001). For example, there are five eIF4E isoforms in *Caenorhabditis elegans* (Keiper et al, 2000), and there are eIF4E-related proteins in *Arabidopsis thaliana* (nCBP; Ruud et al, 1998) and vertebrates (4EHP; Rom et al, 1998). However, here we have shown that eIF4E2 in *S. pombe* represents a new subclass of the wider family of eIF4E-related proteins, one that functions in cellular stress responses. Moreover, the cell upregulates the *tif452* gene in response to at least two types of stress. The upregulated level is evidently required for an optimal stress response, but is still below the level that causes measurable growth inhibition. A recent study of *S. pombe* genes that are implicated in various environmental stresses identified a number of potential regulatory promoter motifs (Chen et al, 2003), including a highly T-rich motif that is thought to target a gene for regulation via the Sty1 protein kinase pathway, but not via its known transcription factor substrate, Atf1. We have now identified a motif of this type in the *tif452* sequence. This suggests that upregulation of *tif452* involves Sty1 together with an as yet unidentified transcription factor.

We have also observed that salt stress causes a reduction in the cellular level of eIF4G, with no detectable effect on eIF4E1 abundance. This may be partly attributable to degradation of eIF4G. However, we cannot rule out the possibility that part of the eIF4G becomes insoluble under such conditions and is then lost in the pellet fraction during preparation of cell extracts (see the Methods section). Comparable effects of stress on eIF4G were reported previously for *Saccharomyces cerevisiae* (Berset et al, 1998) and for human cells (Cuesta et al, 2000). This means that cells that are stressed in this way will have a reduced capacity to perform normal protein synthesis. Yet at the same time, they will have an increased content of a factor (eIF4E2) that seems to be capable of promoting translation of mRNAs with complex leaders. Given this combination of circumstances, we propose a readily

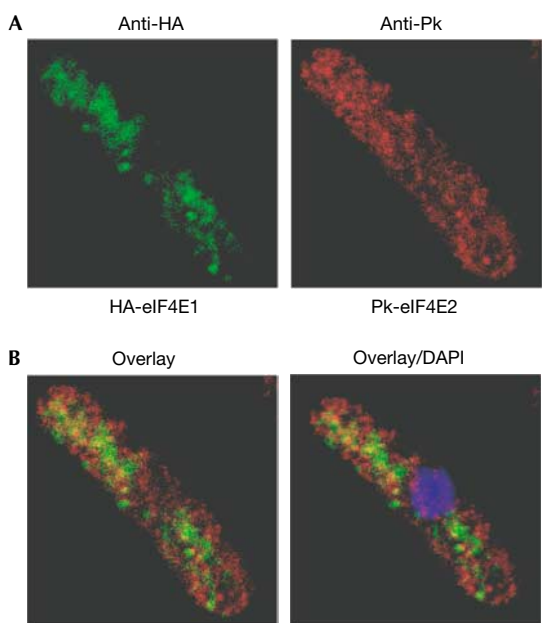


Fig 7 | Subcellular localization of eIF4E1 and eIF4E2. *S. pombe* eIF4E1 and eIF4E2 are differently distributed in the cell cytoplasm. Indirect immunofluorescence of CB12 cells carrying HA-eIF4E1 or Pk-eIF4E2 is shown in (A), and both fusion plasmids are shown in (B). HA-eIF4E1 was visualized using antiserum directed against the HA epitope and FITC-conjugated anti-rabbit secondary antibodies. Pk-eIF4E2 was visualized using anti-Pk epitope and Texas red-conjugated anti-mouse secondary antibodies. Nuclei were stained with DAPI (B). The overlay reveals colocalization in yellow.

testable model for at least some of the post-transcriptional stress responses in *S. pombe* in which eIF4E2 promotes translation via an alternative, as yet undefined, initiation pathway, thus favouring mRNAs the encoded products of which are important for stress resistance (see below).

It has been known for some time that eIF4E proteins can be both cytoplasmic and nuclear in eukaryotic cells (Lejbkiewicz et al, 1992; Lang et al, 1994). In mammalian cells, nuclear eIF4E interacts with the promyelocytic leukaemia (PML) protein in bodies that are distinct from nucleoli (Strudwick & Borden, 2002). In contrast, in *S. pombe* we have observed that both eIF4E1 and eIF4E2 are relatively poorly represented in the nucleus and that they seem to be largely organized into small cytoplasmic bodies. However, this does not rule out the possibility that either, or both, of these proteins may be involved in nucleocytoplasmic transport of mRNA, as we have no information on the dynamics of their movements within the cell. Finally, the marked cytoplasmic bias in the overall distributions of eIF4E1 and eIF4E2 in *S. pombe* seems to suggest that this organism may need less of this type of factor in the nuclear compartment than cells of *S. cerevisiae* or of metazoa. Further work will be required to provide an explanation for this apparent difference.

SPECULATION

The distinct distributions of eIF4E1 and eIF4E2 seen in *S. pombe* are consistent with the association of these factors with separate

Table 1 | Plasmids used in this study

Plasmid	Description	Reference
pREP1	High-copy-number <i>S. pombe</i> expression vector	Maundrell (1993)
pREP1-FLAG eIF4E1	FLAG-tagged <i>tif451</i> in pREP1	This study
pREP1-FLAG eIF4E2	FLAG-tagged <i>tif452</i> in pREP1	This study
pREP41(<i>leu</i>),pREP42(<i>ura</i>)	Middle-copy-number <i>S. pombe</i> expression vectors	Maundrell (1993)
pREP41 <i>LUC</i>	pREP41 carrying <i>LUC</i> gene	This study
pREP41B3 <i>LUC</i>	pREP41 carrying <i>LUC</i> gene with B3 in 5'UTR	This study
pREP41-4E1	<i>tif451</i> in pREP41	This study
pREP42-4E2	<i>tif452</i> in pREP42	This study
HA-eIF4E1	HA-tagged <i>tif451</i> in pREP41	This study
Pk-eIF4E2	Pk-tagged <i>tif452</i> in pREP42	This study

subpopulations of mRNA. At least some of the foci into which eIF4E1 is largely organized are likely to accommodate polysomes translating a large proportion of the *S. pombe* transcriptome (including 'housekeeping' mRNAs) via the standard eukaryotic translation pathway. The organization of eIF4E2 in the cell, conversely, reflects a different function. We speculate that the latter factor functions to support the translation of stress-related mRNAs. There is comparatively little information about the structure of *S. pombe* mRNA leaders, but by analogy to other organisms, we think it is likely that many stress-response genes have distinctive 5'UTRs that could be targeted for relatively efficient translation under stress conditions. A precedent for a selectively translated subset of mRNAs exists in the form of the 5' TOP mRNAs of higher eukaryotes (Meyuhas & Hornstein, 2000). In *S. pombe*, eIF4E2 may have evolved through gene duplication and selection to provide this yeast with an additional mechanism for responding to stress. The proposed mode of action of eIF4E2 can be tested in future work, which will include comparison of the post-transcriptional expression profiles of wild-type and *tif452*Δ strains under different growth conditions and characterization of the leader structures of mRNAs that are found to be subject to *tif452*-dependent translation.

METHODS

Strains, plasmids and growth conditions. The strains used were CB12 [*h⁻ S leu1-32 ura4-D18 ade6-M216*] and the deletion strain CB12*tif452*Δ [*h⁻ S leu1-32 ura4-D18 ade6-M216 tif452::* \ominus *kanMX6*] (Ptushkina et al, 2001). For growth assays, cells were grown overnight and diluted to OD₆₀₀=0.2 and subsequently incubated in yeast extract with supplements (YES) medium (<http://www.bio.uva.nl/pombe/handbook>) under different stress conditions up to OD₆₀₀=1.0. Plasmids used in the present study are listed in Table 1.

RNA analysis. Total RNA was extracted using a standard phenol-chloroform method (Schmitt et al, 1990). Characterization of the 5' and 3' ends of *tif452* mRNA was performed using the SmartTM RACE cDNA Amplification Kit (BD Biosciences). Northern blots were analysed quantitatively using a Typhoon 8600 Imager.

In vivo reporter assays. Cells from 1 ml of culture (OD₆₀₀=0.8–1.0) were harvested, washed and resuspended in extraction buffer

(20 mM HEPES pH 7.6, 100 mM KCl). Cell extracts were prepared by agitation with glass beads, and protease inhibitors (fungal protease inhibitors, Sigma) were added before breakage. A soluble fraction was prepared by centrifugation of the extracts, and used for further analysis. Luciferase activities were determined as described previously (Ptushkina et al, 2001).

Western blots. Aliquots of the cell-free extracts (prepared as above) corresponding to 10 µg of total protein were submitted to 12% SDS-PAGE and blotted by means of semidry transfer to nitrocellulose membranes. Membranes were probed using the appropriate primary antibodies together with horseradish peroxidase-conjugated secondary antibodies. Anti-eIF4E2 polyclonal antibodies were raised in rabbits and used in western blotting experiments at a dilution of 1:5,000. To prevent crossreaction with eIF4E1, the serum was blocked using 1 µg/ml of purified eIF4E1. Rabbit anti-eIF4E1 serum was used at a dilution of 1:2,500.

Fluorescence microscopy. *S. pombe* CB12 cells carrying either HA-eIF4E1, Pk-eIF4E2 or both fusion plasmids (based on pREP41HAN and pREP42PkN vectors; see Table 1) were grown in Edinburgh minimal medium (EMM) (<http://www.bio.uva.nl/pombe/handbook>) with supplements to mid-log phase. The cells were fixed in 3% formaldehyde and 0.2% glutaraldehyde solution and processed as described previously (Hagan & Hyams, 1988). Fixed cells were stained with rabbit polyclonal antibodies that recognize the haemagglutinin (HA) epitope (diluted 1:100, Sigma), followed by FITC-conjugated goat anti-rabbit IgGs (1:100, Vector Laboratories), or with mouse monoclonal antibody SV5-P-k1 that recognizes the Pk epitope of the SV5 P protein (diluted 1:50, Serotec), followed by Texas red-conjugated horse anti-mouse IgGs (1:100, Vector Laboratories). Nuclei were stained with DAPI (1 µg/ml in the mounting medium). Cells were mounted on poly-L-lysine-coated coverslips and analysed using a confocal microscope (Zeiss LSM510) with an HC Plan APO OS × 100 oil-immersed NA 1.4 objective.

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